EFFECT OF STORAGE FOR THREE MONTHS AT DIFFERENT TEMPERATURES ON THE SENSITIVITY TO STREPTOMYCIN AND ISONIAZID OF CULTURES OF TUBERCLE BACILLI.

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V. DEVAKI, K. MOHAN, AND P. R. J. GANGADHARAM.

Reproduced from :

INDIAN JOURNAL OF MEDICAL RESEARCH

Vol. **55,** No, 11, November, 1967.

CAMBRIDGE PRINTING WORKS
DELHI
1967

Ind. Jour. Med. Res., **55,** 11, November, 1967, pp. 1150-1158.

EFFECT OF STORAGE FOR THREE MONTHS AT DIFFERENT TEMPERATURES ON THE SENSITIVITY TO STREPTOMYCIN AND ISONIAZID OF CULTURES OF TUBERCLE BACILLI.

V. DEVAKI, K. MOHAN, AND P.R.J. GANGADHARAM.

(From the Central Laboratory, Indian Council of Medical Research Drug Resistance Survey, Tuberculosis Chemotherapy Centre, Madras-31, India.)

[Received for publication, June 13, 1967.]

INTRODUCTION.

MAINTENANCE of bacterial strains by repeated subcultivation is both expensive, laborious and time-consuming ; moreover, there is always the possibility of contamination or of differential selection of sub-strains with specific properties. In consequence, several methods, such as freeze-drying or storage at low temperatures, have been introduced by which bacterial cultures can be kept alive for long periods with their reproductive and metabolic activity at an extremely low level. However, information is rather sparse on such methods for the storage of tubercle bacilli. For instance, Corper and Gauss (1923) found that tubercle bacilli remained viable in Petroff's egg medium or glycerol agar after storage in the incubator or refrigerator for 4 to 8 months. Later workers (Heckly, 1950 ; Stern and Tompsett, 1951 ; Jones, 1957 ; Tsukamura, 1965) suggested preservation of cultures by freezing them in various diluents. More recently, Tarshis (1961) compared storage of cultures in various diluents at –20°C. and concluded that, with minor exceptions, most types of mycobacteria (including tubercle bacilli) can be stored for at least 3 years without any major change in their viability or drug resistance. However, these procedures are time-consuming, expensive and require special equipment and are? therefore, not very practicable in developing countries with limited resources.

Our attention was drawn to this problem of storage of cultures during the course of the first drug resistance survey undertaken by the Indian Council of Medical Research (in preparation). In this survey, the Central Laboratory at Madras had to undertake bacteriological examinations. including drug sensitivity tests, on a total of 2,700 specimens of sputum sent from each of the 9 centres distributed in various parts of India. Sensitivity tests were set up on nearly all the positive cultures *within 2 or 3 days of their becoming positive.* However, the organizational problems encountered were considerable and often acute. This experience prompted the present investigation on the effect of storage at different temperatures on the viability and sensitivity to streptomycin and isonazid of cultures of tubercle bacilli. The period of storage was chosen to be 3 months as this represents, in our opinion, a reasonable period in which an equitable distribution of the work-load can be arranged in any large-sized laboratory.

MATERIALS AND METHODS.

Consecutive specimens of sputum with a positive smear result by fluorescence microscopy (Holst, Mitchison and Radhakrishna, 1959) were obtained over a period of 6 days from patients attending the Tuberculosis Chemotherapy Centre, Madras. After decontamination with sodium hydroxide, each sputum concentrate was inoculated onto 5 slopes of Lowenstein-Jensen medium without potato starch (Jensen, 1955). The slopes were arranged in rows of five in a rack and incubated at 37°C. and read every week for 8 to 9 weeks.

Design of the experiment :

At the end of 8 to 9 weeks :-

(a) sensitivity tests to streptomycin and isoniazid were set up (by one of the two technicians) from the growth on the *first* positive slope in each row ;

(b) the second positive slope in each row was stored at –20°C. in the deep-freeze ;

(c) the third at 37°C. in the incubator ;

(d) the fourth at 4°C. in the laboratory cold-room, and

(e) the fifth at approximately 28°C. on the laboratory bench in a closed cardboard container.

If growth was present on only 4 slopes, the effect of storage at 28°C. was not studied, and if it was present on only 3 slopes, the effect of storage at 4°C. was also not investigated. If less than 3 slopes yielded growth, they were discarded. This pattern of selection was adopted so that the maximum amount of information regarding the effects of storage at –20°C. and 37°C.–the two most commonly used methods (Willis and Cummings, 1952 ; Tarshis, *loc. cit.*– would be obtained.

At the end of 3 months of storage, for each specimen, sensitivity tests to streptomycin and isoniazid were set up, without subcultivation, from the growth on slopes, *(b), (c), (d)* and *(e)* by the *same* technician who had set up the test from the growth on slope *(a)* for that particular specimen. All the slopes meant for each technician were put together and randomized by a statistician (K.M.). The sensitivity test results were *all* read by one of us (V.D.). Neither this reader nor the technicians knew the identity of any individual slope at any stage of the investigation.

Procedure for sensitivity tests.– The procedures adopted for sensitivity tests to streptomycin and isoniazid were the same as those employed by the Tuberculosis Chemotherapy Centre, Madras (1959). The drug concentrations (µg./ml.) used were :

Drug	*Test strain*	*H37Rv*
Streptomycin	4, 16, 32, 64, 256	1, 2, 4, 8
Isoniazid	0.2, 1, 5, 50	0.025, 0.05, 0.1,0.2, 1

The results of all the tests were read at the end of 4 weeks incubation at 37°C. Isoniazid sensitivity was expressed as the minimal inhibitory concentration (ICM)

inhibiting growth of 20 colonies or more and streptomycin sensitivity as a resistance ratio (RR), i.e. the ratio of the MIC of the test strain to the MIC of the standard strain, H37Rv.

Numbers in the present analysis.– In all, cultures were set up from each of 110 consecutive specimens with a positive smear result. However, 15 of these specimens have been excluded from further consideration for the following reasons :--

Positive on none of the 5 slopes	...	8 specimens
Positive on only 1 or 2 slopes	...	5 specimens
Sensitivity test before storage not set up	..	2 specimens

Of the remaining 95 specimens, 60 yielded growth on all 5 slopes (and so are included in all the investigations), 23 on 4 slopes and 12 on 3 slopes. One positive slope was stored in the cold-room in error instead of in the deep-freeze, and sensitivity tests were not set up on 2 positive slopes that had been stored on the laboratory bench for 3 months. Consequently, the numbers in the analyses below are 94 for deep-freeze storage, 83 for cold-room storage, 58 for storage on the laboratory bench, and 95 for incubator storage.

RESULTS.

Viability of tubercle bacilli.– Of 95 positive cultures that were stored at 37°C., growth was obtained after storage from only 66 (69 per cent) (Table I). The corresponding proportions were 97 per cent of 58 for storage at 28°C., 99 per cent for 83 for storage at 4°C. and 93 per cent of 94 for storage at –20°C. ; the differences between these proportions are statistically non-significant but all three proportions are significantly higher than the survival rate of cultures stored at 37°C., namely 69 per cent ($P<0.005$). Thus, storage for 3 months at 37°C. effected adversely the viability of tubercle bacilli. Further analyses (not tabulated here) did not show any association between the loss of viability due to storage at 37°C. and *(a)* the degree of smear and culture positivity before storage of the specimens *(b)* the period of incubation after which the initial culture (before storage) was reported as positive, and (c) the streptomycin and isoniazid sensitivity of the culture before storage.

TABLE I.

Viability of cultures after storage for three months, according to temperature of storage.

Place of storage.	Storage temperature (centigrade).	Number of cultures stored.	CULTURES THAT WERE VIABLE AT 3 MONTHS:	
			Number.	Per cent.
Deep-freeze	—20°	94	87	*93*
Gold-room	4°	83	82	*99*
Laboratory bench	28°	58	56	*97*
Incubator	37°	95	66	*69*

Streptomycin sensitivity.– In Table II, the results of the streptomycin sensitivity test after storage of the culture is related to the results before storage. Identical results

TABLE II.

Effect of storage at different temperatures on the streptomycin sensitivity of cultures of Tubercle bacilli.

Result after storage in relation to the result before storage	Place of storage: Deep-freeze (— 20°C.)		Cold-room (4°C.)		Lab. bench (28°C.)		Incubator (37°C.)	
	Number of cultures.	Per cent.	Number of cultures.	Per cent.	Number of cultures.	Per cent.	Number of cultures.	Per cent.
(a) Identical	38	*44*	36	*44*	22	*39*	21	*32*
(b) Lower	31	*36*	31	*38*	25	*45*	37	*56*
(c) Higher	18	*21*	15	*18*	9	*16*	8	*12*
Total ..	87	*101*	82	*100*	56	*100*	66	*100*
(b) — (c)	15%		20%		29%		44%	
χ^2	3·45		5·57		7·53		18·69	
p*	0·06		0·02		<0·01		<0·0001	

*This represents the probability that the observed difference (or a larger one) between the results before storage and those after storage [in essence, the contrast between terms (b) and (c)] could have occurred due to sampling fluctuations.

from the same specimen were obtained in the test before and after storage in 44 per cent of 87 cultures stored at –2O°C., 44 per cent of 82 cultures stored at 4°C., 39 per cent of 56 stored at 28°C. and 32 per cent of 66 stored at 37°C. The sensitivity test result after storage was lower in 36 per cent of the cultures stored at –20°C. and higher in 21 per cent ; the contrast bordering on statistical significance (P = 0.06) ; the corresponding proportions were 38 and 18 per cent for cultures stored at 4°C. (P=0.02), 45 and 16 per cent for those stored at 28°C. (P <0.01) and 56 and 12 per cent for those stored at 37°C. (P < 0.000l), respectively.

In short, the storage of cultures for a period of 3 months, whether in the deep-freeze, cold-room, laboratory bench or the incubator, resulted more often in lower resistance ratios (RR) after storage [see row (6) in Table II] than higher ones [row (c)] ; the difference between these two percentages, which is a measure of the change in sensitivity due to storage, increased with storage temperature ; thus, for storage temperature of –2O°C., 4°C., 28°C.. and 37°C., the differences were 1.5, 20, 29 and 44 per cent, respectively.

Classification of cultures as sensitive or resistant.– In view of the above findings, it is important to consider the extent to which storage affects the classification of cultures as streptomycin-sensitive or resistant, since errors in such a classification are likely to have serious clinical implications for patients receiving chemotherapy which includes streptomycin. For this purpose, cultures with a resistance ratio of less than 4 have been regarded as sensitive and those with a resistance ratio of 4 or more as resistant. However. cultures with a resistance ratio of 16/4, either before storage or

after storage, had to be excluded from this analysis on account of uncertainty regarding their classification as sensitive or resistant. This uncertainty was caused by the fact that an 8 µg./ml. concentration was not employed in the present study ; if it had been employed, it is possible that some of *these* cultures would have had a resistance ratio of 8/4 and, therefore, been classsified as sensitive, whereas all of them would now have to be classified as resistant.

The proportion of cultures whose classification as sensitive or resistant was unaffected by storage at –20°C. was 95 per cent. the corresponding proportions being 90 per cent for storage at 4°C., 92 per cent for storage at 28°C. and 88 per cent for storage at 37°C (Table III). Among cultures with contrary classifications, resistance was less frequently reported after storage. Thus, for storage at –20°C., while 3 cultures were classified as resistant before storage but sensitive after storage, only one culture was classified as sensitive before storage but resistant after storage. The corresponding numbers were 5 and 2 for storage at 4°C., 4 and 0 for storage at 28°C., and 5 and 2 for storage at 37ºC., respectively.

TABLE III.

Effect of storage at different temperatures on the classification of cultures as streptomycin-sensitive or streptomycin-resistant.

STREPTOMYCIN SENSITIVITY:		PLACE OF STORAGE:							
		Deep-freeze (– 20°C.)		Cold room (4°C.)		Lab. bench (28°C.)		Incubator (37°C.)	
Before storage.	After storage.	Number of cultures.	Per cent.	Number of cultures.	Per cent.	Number of cultures.	Per cent.	Number of cultures.	Per cent.
Sensitive	Sensitive	55	*95*	47	*90*	34	*92*	38	*88*
Resistant	Resistant	19		16		12		12	
Resistant	Sensitive	3	*4*	5	*7*	4	*8*	5	*9*
Sensitive	Resistant	1	*1*	2	*3*	0	*0*	2	*4*
	Total ..	78	*100*	70	*100*	50	*100*	57	*101*
Number of cultures excluded from comparison*		9	..	12	..	6	..	9	..

*** On account of uncertainty in the interpretation of their sensitivity test results (see text)**

Of 4 batches in which all the sensitivity tests before storage were set up, 3 had an MIC of 8 µg./ml. for the standard sensitive strains H37Rv and one an MIC of 4 µg./ml., as compared with 13 and 2, respectively, among 15 batches in which all the sensitivity tests after storage were set up. Thus, it seems unlikely that the decrease in streptomycin resistance reported above was due to variation in standards during the period of the study.

Isoniazid sensitivity.– The test result after storage was the same as that before storage in 88 per cent of 86 cultures stored at –20°C., 85 per cent of 82 stored at

4°C., 91 per cent of 56 stored at 28°C. and 82 per cent of 66 stored at 37°C. (Table IV). These percentages are likely to be over-estimates of the extent of agreement between the test results before and after storage since they include 58, 52, 48 and 58 per cent, of cultures, respectively, that were inhibited, both before storage and after storage, by 0.2 µg./ml. of isoniazid (the lowest concentration employed in the present study) ; obviously, if concentrations lower than 0.2 µg./ml. had also been employed, the proportion of cultures with identical results before and after storage would, in all likelihood, have decreased. The result after storage was lower than that before storage in 6 per cent of the cultures stored at –20°C. and higher in 6 per cent; the corresponding proportions were 10 and 5 per cent for cultures stored at 4°C., 5 and 4 per cent for those stored at 28ºC., and 12 and 6 per cent for those stored at 37°C respectively.

TABLE IV.

Effect of storage at different temperatures on the isoniazid sensitivity of cultures of Tubercle bacilli.

Result after storage in relation to the result before storage.	PLACE OF STORAGE :							
	Deep-freeze (—20°C).		Cold-room (4°C).		Lab. bench (28°C).		Incubator (37°C).	
	Number of cultures	Per cent.	Number of cultures.	Per cent.	Number of cultures.	Per cent.	Number of cultures.	Per cent.
Identical*	76	*88*	70	*85*	51	*91*	54	*82*
Lower	5	*6*	8	*10*	3	*5*	8	*12*
Higher	5	*6*	4	*5*	2	*4*	4	*6*
Total	**86**	*100*	82	*100*	56	*100*	66	*100*

*** Including cultures that were inhibited, both before storage and after storage, by 0.02 µg./ml., the lowest isoniazid concentration employed in the present investigation (see text).**

Next, defining cultures with an MIC of 1 µg./ml. or more as resistant, the effect of storage for 3 months on the classification of cultures as isoniazid-sensitive or isoniazid-resistant was studied (Table V), The classification was unaffected in the very great majority of cultures, irrespective of the storage temperature. Thus, the proportions with the same classification before and after storage were 94, 95, 98 and 95 per cent for cultures stored at –20ºC., 4ºC., 28°C. and 37ºC., respectively. In the remaining, there was no evidence of any systematic change in sensitivity due to storage ; it is, therefore, likely that the discrepancies observed are due to the inherent errors in the test-that is, unrelated to whether or not the culture was stored for 3 months.

As with streptomycin, the standards were reasonably stable over the period of study. Thus, of the 4 batches in which all the sensitivity tests before storage were set

TABLE V.

Effect of storage at different temperatures on the classification of cultures as isoniazid-sensitive or isoniazid-resistant.

ISONIAZID SENSITIVITY :		PLACE OF STORAGE:							
		Deep-freeze (—20°C).		Cold-room (4°C).		Lab. bench (28°C).		Incubator (37°C).	
Before storage.	After storage.	Number of cultures.	Per cent.	Number of cultures.	Per cent.	Number of cultures.	Per cent.	Number of cultures.	Per cent.
Sensitive	Sensitive	50	*94*	43	*95*	27	*98*	38	*95*
Resistant	Resistant	31		35		28		25	
Resistant	Sensitive	3	*3*	2	*2*	0	*0*	3	*5*
Sensitive	Resistant	2	*2*	2	*2*	1	*2*	0	*0*
Total		86	*99*	82	*99*	56	*100*	66	*100*

up, one had an MIC of 0.05 µg./ml. for the H37Rv strain and three an MIC of 0.1 µg/ml. ; correspondingly, of the 15 batches in which the cultures were tested after storage, the MIC for H37Rv was 0.025 µg./ml. in one, 0.05 µg/ml. in two and 0.1 µg./ml. in the remaining 12.

In short, the storage of cultures for 3 months does not seem to have had any effect on the isoniazid sensitivity of the tubercle bacilli ; however, it would be desirable to confirm this conclusion on a larger number of cultures and more closely-spaced isoniazid concentrations.

DISCUSSION.

This paper reports the findings of an investigation undertaken to study the effect of storage, for 3 months at various temperatures, on the viability and on the drug sensitivity of cultures of tubercle bacilli. Sensitivity tests were set up for each of 95 sputum specimens, both from the fresh culture and from replicate culture slopes of the same specimen after storage for 3 months in the deep-freeze (–20°C.), cold room (4°C.), laboratory bench (28°C.) or the incubator (37°C.).

Of the 95 positive cultures stored at 37°C. in the incubator, 29 (31 per cent) did not grow after storage as compared with 3 per cent of 58 stored at 28°C., 4 per cent of 83 stored at 4°C., and 7 per cent of 94 stored at -20°C.; all three contrasts were significant ($P < 0.05$). The greater loss of viable organisms in the incubator-stored cultures might have been caused by the exhaustion of the available nutrients in the medium or by the autolysis of the bacteria ; both possibilities are likely since bacterial proliferation is maximal at 37°C. Loss of viability on account of storage in an incubator has also been reported by Heckly *(loc. cit.)*, who used Tween- albumin liquid medium ; however, Corper and Gauss *(loc. cit.)* reported no loss of viability over 4 to 8 months using Petroff's medium or glycerol agar.

Considering the outcome of streptomycin sensitivity test results, there was a greater tendency for the result after storage to be lower (rather than higher) than that before storage. This apparent 'decrease in streptomycin resistance' was greater with the higher storage temperatures investigated ; it bordered on statistical significance for cultures stored at –20°C. ($P = 0.06$) and was clearly significant for cultures stored at 4°C. ($P = 0.02$), at 28°C. ($P < 0.01$) and at 37°C. ($P < 0.0001$). The behaviour of the control strain H37Rv, which was set up in every batch of tests, did not suggest any change in laboratoy standards during the course of the study. It, therefore, seems reasonsable to consider other explanations for this finding of 'loss of resistance'. Possibly, a selective and differentially higher mortality of streptomycin-resistant mutants during storage can account for this finding.

Our findings differ from those of Heckly *(loc. cit.),* Stern and Tompsett *(loc. cit.),* Jones *(loc. cit.),* Tarshis (1961) and Tsukamura *(loc. cit.)* in that none of them observed a decrease in the mean streptomycin sensitivity after storage in a frozen state for periods ranging from 4 to 36 months. However, in these studies, liquid medium was used with specific diluents and protective colloids, in contrast to the present one where solid Lowenstein-Jensen medium with no specific additives was used.

As regards isoniazid sensitivity, there was no evidence of any systematic change as a result of storage. Indeed, the classification of cultures as sensitive or resistant was the same before and after storage in at least 94 per cent of the cases for all the four temperatures investigated. It must, however, be emphasized that the number of cultures studied was not very large, the isoniazid concentrations were not very closely spaced and no concentration lower than 0.2 µg./ml. was employed ; the conclusion above must, therefore, be accepted with some caution.

From the findings in the present study, it is possible to make some tentative recommendations. The first is that storage of cultures at 37°C. (incubator temperature) for periods of 3 months or longer should definitely be avoided since, in the present study, nearly one-third of the cultures failed to grow after storage. Further. if streptomycin sensitivity is the characteristic under assessment, it is inadvisable to store cultures for as long as 3 months since such a measure would result in under-estimating streptomycin resistance ; however, if circumstances necessitate such a measure, storage in the deep-freeze (–20°C.) or, alternatively, in a refrigerator (at 4°C.) may be recommended. Finally, since isoniazid sensitivity was hardly affected by storage for 3 months, cultures awaiting such tests may be stored on the laboratory bench (i.e. at approximately 28°C.) for periods of up to 3 months.

SUMMARY.

Tests for sensitivity to streptomycin and isoniazid were undertaken, immediately and after 3 months of storage at different temperatures (–20°C., 4°C., 28°C., and 37°C.), on replicate culture slopes from each of 95 sputum specimens. About one third of the cultures failed to grow after storage at 37°C., as compared with 7, 1 and 3 per cent for storage at –20°C., 4°C. and 28°C. respectively. The storage resulted in

a decrease in streptomycin resistance, the decrease being larger for the higher storage ‘temperatures. No change in isoniaiid sensitivity was observed.

The authors are grateful to Sri C. Jayaraman and Sri V. Ramamurthy for technical assistance, and to Dr. S. Radhakrishna, who gave them invaluable assistance and statistical advice in the analysis of results, and preparation of the report.

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